

## Short Report: Phylogenetically Distinct Hantaviruses in the Masked Shrew (*Sorex cinereus*) and Dusky Shrew (*Sorex monticolus*) in the United States

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**Abstract.** A limited search for hantaviruses in lung and liver tissues of *Sorex* shrews (family *Soricidae*, subfamily *Soricinae*) revealed phylogenetically distinct hantaviruses in the masked shrew (*Sorex cinereus*) from Minnesota and in the dusky shrew (*Sorex monticolus*) from New Mexico and Colorado. The discovery of these shrew-borne hantaviruses, named Ash River virus and Jemez Springs virus, respectively, challenges the long-held dogma that rodents are the sole reservoir hosts and forces a re-examination of their co-evolutionary history. Also, studies now underway are aimed at clarifying the epizootiology and pathogenicity of these new members of the genus *Hantavirus*.

Based on phylogenetic analyses of full-length viral genomic sequences and host mitochondrial DNA (mtDNA) sequences, hantaviruses segregate into clades that parallel the evolution of murinae, arvicolinae, neotominae, and sigmodontinae rodents.<sup>1–4</sup> Whether insectivores (or soricomorphs), which are sympatric with rodents, are involved in the evolutionary origins of hantaviruses has not been systematically studied, despite previous reports of hantavirus antigens in tissues of the Eurasian common shrew (*Sorex araneus*), alpine shrew (*Sorex alpinus*), Eurasian water shrew (*Neomys fodiens*), and common mole (*Talpa europea*) captured in Russia<sup>5,6</sup> and the former Yugoslavia.<sup>7</sup> Also, the isolation of Thottapalayam virus (TPMV), from the Asian house shrew (*Suncus murinus*) in India,<sup>8–10</sup> would suggest that soricids might serve as legitimate reservoir hosts of hantaviruses.

Armed with the newly acquired full genome of TPMV and emboldened by our recent discovery of genetically distinct hantaviruses in soricine shrews, including Camp Ripley virus (RPLV) in the northern short-tailed shrew (*Blarina brevicauda*) in the United States<sup>11</sup> and Cao Bang virus (CBNV) in the Chinese mole shrew (*Anourosorex squamipes*) in Vietnam,<sup>12</sup> we launched a small-scale search for soricid-borne hantaviruses by accessing the archival tissue collection of *Sorex* (family *Soricidae*, subfamily *Soricinae*), housed in the Museum of Southwestern Biology at the University of New Mexico.

RNA extracts, from 100 mg each of frozen lung or liver tissues from the masked shrew (*S. cinereus*), dusky or montane shrew (*S. monticolus*), dwarf shrew (*S. nanus*), northern water shrew (*S. palustris*), Trowbridge shrew (*S. trowbridgii*), tundra shrew (*S. tundrensis*), and vagrant shrew (*S. vagrans*), captured in the United States between 1983 and 2005 (Table 1), were analyzed for hantavirus sequences by reverse transcription-polymerase chain reaction (RT-PCR). The remarkably divergent genomes of soricid-borne hantaviruses presented challenges in designing suitable primers, but we eventually succeeded in amplifying regions of the S (outer OSM55: 5'-TAGTAGTAGACTCC-3' and HTN-S6: 5'-AGCTCIG-

GATCCATITCATC-3'; inner Cro2R: 5'-AIGAYTGRTAR-AAIGAIGAYTTYTT-3' and PHS-5F: 5'-TAGTAGTAG-A CTCCTTRAARAGC-3')<sup>13,14</sup> and L (outer HAN-L-F1: 5'-ATGTAYGTBAGTGCWGTATGC-3' and HAN-L-R1: 5'-AACCADTCWGTGCCATC-3'; inner HAN-L-F2: 5'-TGCWGTGCHACIAARTGGTC-3' and HAN-L-R2: 5'-GCRTCRCWGARTGRTGDGCA A-3')<sup>14</sup> segments.

First- and second-round PCR were performed in 20- $\mu$ L reaction mixtures, containing 250  $\mu$ M dNTP, 2 mM MgCl<sub>2</sub>, 1 U of AmpliTaq polymerase (Roche, Basel, Switzerland), and 0.25  $\mu$ M of each primer. Initial denaturation at 94°C for 2 min was followed by two cycles each of denaturation at 94°C for 30 sec, two-degree step-down annealing from 46°C to 38°C for 40 sec, and elongation at 72°C for 1 min, then 30 cycles of denaturation at 94°C for 30 sec, annealing at 42°C for 40 sec, and elongation at 72°C for 1 min, in a GeneAmp PCR 9700 thermal cycler (Perkin-Elmer, Waltham, MA). PCR products were separated by agarose gel electrophoresis and purified using the Qiaex Gel Extraction Kit (Qiagen, Hilden, Germany). DNA was sequenced directly using an ABI Prism 377XL Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were then processed using the Genetyx version 6 software (Genetyx Corporation, Tokyo, Japan) and aligned using Clustal W<sup>15</sup> and transAlign.<sup>16</sup> For phylogenetic analysis, maximum-likelihood consensus trees were generated by the Bayesian Metropolis–Hastings Markov Chain Monte Carlo (MCMC) tree-sampling methods as implemented by Mr. Bayes using a GTR+I+G model of evolution, as selected by Modeltest v.3.7, partitioned by codon position.<sup>17–19</sup>

Of the 54 *Sorex* shrews studied, hantavirus S- and L-segment sequences were detected in *S. cinereus*, captured near the Ash River Station in Voyageur's National Park (48°26'N, 92°00'W) in St. Louis County, Minnesota, in August 1994, and in Chippewa National Forest in Cass County, Minnesota, in July 1983, as well as in *S. monticolus*, captured near Jemez Springs (35°48'N, 106°30'W) in Sandoval County, New Mexico, in September 1996, September 1998 and September 2000, and along Trapline ECJ (40°27'N, 106°00'W) in Jackson County, Colorado, in July 1994 (Table 1). The newly identified hantavirus sequences were designated Ash River virus (ARRV) and Jemez Springs virus (JMSV), respectively. Host identification was verified by morphologic assessment of voucher specimens and by mtDNA sequence analysis of a

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TABLE 1  
RT-PCR detection of hantavirus sequences in tissues of *Sorex* shrews

Genus species	State	County*	Trapping year	Number tested	Number positive
<i>Sorex cinereus</i>	Alaska	Anchorage	1998	10	0
		Anchorage	2005	3	0
	Minnesota	Cass	1983	11	1
		St. Louis	1994	1	1
		Swain	1994	1	0
<i>Sorex haydeni</i>	New Mexico	Sandoval	1996	1	1
<i>Sorex monticolus</i>	Alaska	Anchorage	1998	1	0
		Colorado	Jackson	1994	1
	New Mexico	Sandoval	1996	5	2
		Sandoval	1998	4	1
		Sandoval	2000	4	1
	Utah	Catron	1995	1	0
		Tooele	1997	1	0
		Wasatch	1994	1	0
		Colorado	Hinsdale	1994	1
	<i>Sorex nanus</i>	Colorado	Hinsdale	1994	1
<i>Sorex palustris</i>	Minnesota	Morrison	1999	1	0
	New Mexico	Sandoval	1996	1	0
<i>Sorex trowbridgii</i>	Washington	Kitsap	1996	1	0
<i>Sorex tundrensis</i>	Alaska	Anchorage	2005	2	0
<i>Sorex vagrans</i>	New Mexico	Otero	1994	1	0
		Santa Fe	1996	2	0

\* No counties exist in Alaska, so localities are reported by quadrangle map.

756-base pair region of the cytochrome *b* gene, using previously described universal primers.<sup>20</sup>

Pairwise alignment and comparison of a 1,083-nucleotide region of the S segment and 347-nucleotide region of the L segment showed low sequence similarities between JMSV and representative rodent-associated hantaviruses, of 59.0–62.1% and 60.2–73.8%, respectively (Table 2). Nucleotide sequence differences in this region of the S and L segments among JMSV strains from Sandoval County in 1996, 1998, and 2000 were 0.4–0.8% and 1.1–6.6%, respectively; amino acid sequences differed by 0.6% and 0.9%, respectively. In the L segment, the JMSV strains from New Mexico and Colorado differed by 11.2–12.1% at the nucleotide level but were identical at the amino acid level.

ARRV, from *S. cinereus* captured in Minnesota, showed a similar degree of sequence divergence in the S and L segments from rodent-borne hantaviruses, with the highest homology to Hantaan virus (HTNV) 76–118 (62.7% and 72.1%) and Dobrava virus (DOBV) Greece (62.2% and 73.5%). When the S- and L-segment sequences were compared with soricid-borne hantaviruses, ARRV was more similar to JMSV (71.9% and 75.8%) and CBNV (68.8% and 78.1%), than to TPMV (54.4% and 64.0%) and Tanganya virus (TGNV) (64.4% and 73.8%), a hantavirus detected recently in the Therese shrew (*Crocidura theresae*) in Guinea.<sup>21</sup> At the amino acid level, ARRV and JMSV were 77.5% and 94.0% identical in the partial S and L segments, respectively (Table 2).

Hantavirus S- and L-segment nucleotide sequences, detected in a shrew, which was initially identified as *S. cinereus*, were only 77.5% and 79.5% similar to ARRV, respectively, but 99.1–99.6% and 93.7–99.1% similar to JMSV. Amino acid sequence similarities were 99.1–99.6% and 99.2–100%. This shrew was captured in Sandoval County, New Mexico, at the identical site and time as *S. monticolus* in which JMSV was detected. On mtDNA analysis, this shrew was shown to be *S. haydeni* (not *S. cinereus*). Isolation of JMSV from *S. monticolus* and subsequent plaque-reduction neutralization tests will be necessary to definitively prove that *S. monticolus* is the true reservoir host of JMSV.

Phylogenetic analysis based on partial S- and L-segment sequences, generated by the maximum-likelihood method using Bayesian tree-sampling, placed JMSV and ARRV in a distinct group that included CBNV (Figure 1). This clade was most closely related to that of the murinae rodent-borne hantaviruses. By contrast, the relationship of JMSV and ARRV to TPMV and TGNV, two hantaviruses harbored by crocidurine shrews, varied according to the S- and L-segment data (Figure 1). Similar topologies, supported by bootstrap analysis, were obtained using the neighbor-joining method. Also, amino acid sequence phylogenies, constructed using Tree Puzzle<sup>22</sup> and the BLOSUM 62 model of evolution with a gamma rate-heterogeneity parameter, yielded nearly identical topologies (data not shown).

The masked shrew is the most widely distributed shrew in North America, extending from Alaska and Canada, across Washington, Idaho, south-central Utah, north-central New

TABLE 2  
Nucleotide and amino acid sequence similarities (in percent) of the partial S and L segments between JMSV strain MSB89332 and other hantaviruses

Virus*	Strain	S Segment		L Segment	
		1083 nt	356 aa	347 nt	115 aa
HTN	76-118	62.1	60.5	72.1	78.4
DOB	Greece	62.0	58.0	73.8	79.3
SEO	HR80-39	61.9	58.7	69.7	75.9
SAN	SA14	60.7	62.0	69.7	78.4
PUU	Sotkamo	61.0	54.7	62.3	65.5
TUL	M5302v	59.9	54.9	60.2	63.8
PH	PH-1	59.7	53.6	67.4	67.8
SN	NMH10	59.0	53.1	65.7	64.7
AND	Chile 9717869	60.4	54.5	64.3	65.5
RPL	MSB89863	NA	NA	69.5	75.9
CBN	TC-3	70.1	73.9	76.4	88.8
ARR	MSB73418	71.9	77.5	75.8	94.0
TGN	Tan826	63.1	64.0	68.0	76.7
TPM	VRC-66412	53.0	42.7	63.1	62.1

nt = nucleotide; aa = amino acid; NA = not available.  
\* AND, Andes; ASR, Ash River; CBN, Cao Bang; DOB, Dobrava; HTN, Hantaan; PH, Prospect Hill; PUU, Puumala; RPL, Camp Ripley; SAN, Sangassou; SEO, Seoul; SN, Sin Nombre; TGN, Tanganya; TPM, Thottapalayam; TUL, Tula.

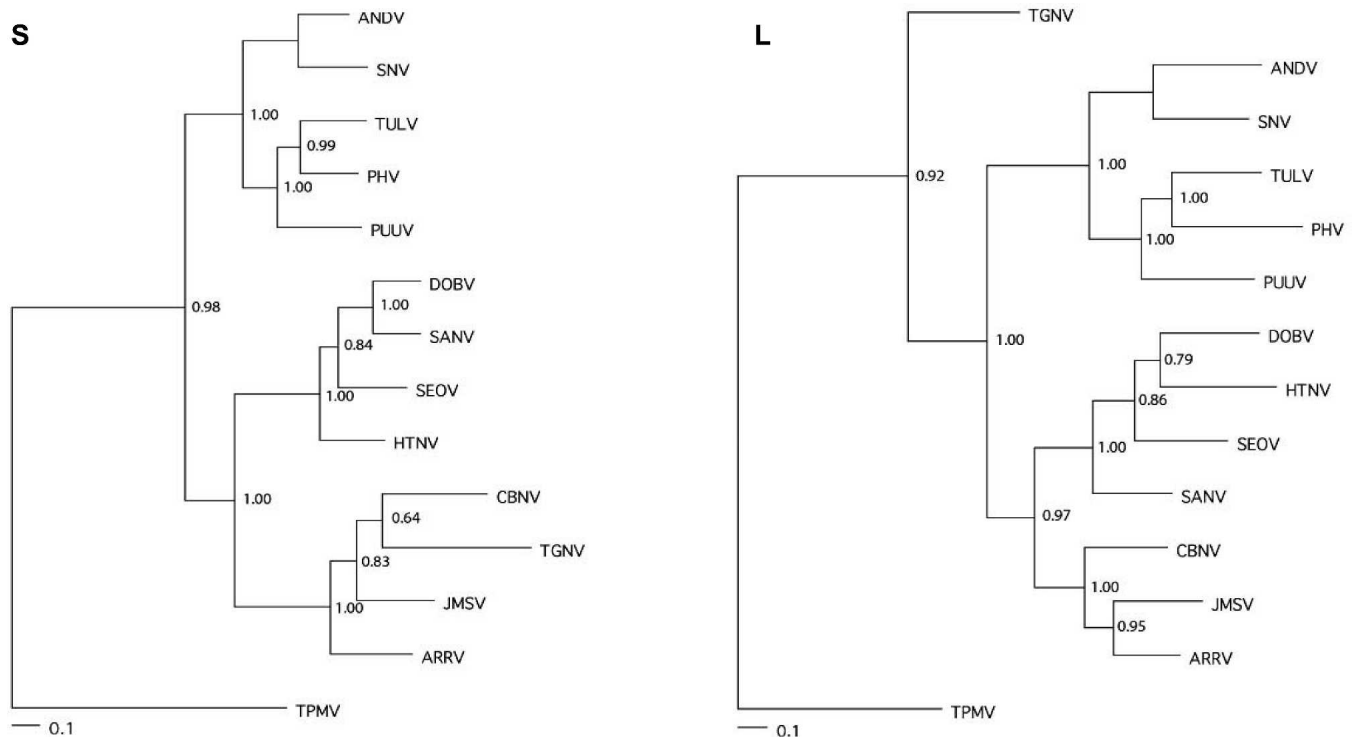


FIGURE 1. Maximum-likelihood phylogenetic consensus trees, generated by Bayesian Metropolis–Hastings Markov Chain Monte Carlo (MCMC) tree-sampling methods using a GTR+I+G model of evolution partitioned by codon position, based on the alignment of the partial 1048-nucleotide S- and 347-nucleotide L-genomic segments of Jemez Springs virus (JMSV MSB89332, EF619960, EF619962) from the dusky shrew (*Sorex monticolus*) and Ash River virus (ARRV MSB73418, EF619961, EF650086) from the masked shrew (*Sorex cinereus*), as well as representative murinae rodent-borne hantaviruses, including Hantaan virus (HTNV 76-118, NC\_005219, NC\_005222), Sangassou virus (SANV SA14, DQ268651, DQ268652), Dobrava virus (DOBV Greece, NC\_005234, NC\_005235), and Seoul virus (SEOV 80 39, NC\_005237, NC\_005238); arvicoline rodent-borne hantaviruses, including Tula virus (TULV M5302v, NC\_005228, NC\_005226), Prospect Hill virus (PHV PH-1, X55128, EF646763) and Puumala virus (PUUV Sotkamo, NC\_005223, NC\_005225); and sigmodontinae and neotominae rodent-borne hantaviruses, including Andes virus (ANDV Chile 9717869, NC\_003467, NC\_003468) and Sin Nombre virus (SNV NMH10, NC\_005215, NC\_005217). Cao Bang virus (CBNV TC-3, EF543524, EF543525) from the Chinese mole shrew (*Anourosorex squamipes*), Tanganya virus (TGNV Tan826, EF050454, EF050455) from the Therese shrew (*Crocidura theresae*), and Thottapalayam virus (TPMV VRC-66412, AY526097, EU001330) from the Asian house shrew (*Suncus murinus*) are also shown. Numbers at each node are posterior node probabilities based on 30,000 trees: two replicate MCMC runs consisting of four chains of 2 million generations each sampled every 100 generations with a burn-in of 5000 (25%). Sufficiency of chain length was determined based on convergence of likelihood values, giving effective sample sizes well over 400 (implemented in MrBayes v. 3). The scale bar indicates 0.1 nucleotide substitutions per site. Alternate phylogenetic methods gave rise to essentially identical topologies, with only minor, unsupported differences. Host identifications of *S. monticolus* and *S. cinereus* were confirmed by mitochondrial DNA sequencing (data not shown).

Mexico, and Nebraska, and throughout the Appalachians in the east. Also among the most common shrews in North America, the dusky shrew is found in Alaska and western Canada, extending southerly through Washington, Idaho, Montana, Utah, Colorado, New Mexico, and Arizona to Mexico. Both species (subfamily *Soricinae*) occupy a variety of moist habitats, as well as grassy areas near streams or rivers; meadows; thickets of willow and alder; spruce-fir forests; and alpine tundra.

Dusky shrews occasionally co-exist with as many as four other soricid species, including masked shrews. As such, sharing of nesting materials, as well as inter- and intra-specific fighting and wounding, might result in hantavirus spillover. The high degree of sequence similarity between hantaviruses detected in *S. monticolus* and *S. haydeni* in Sandoval County, New Mexico, may be instructive. Based on its dominant position in this community, the natural host of JMSV was assigned to *S. monticolus*.

Using parsimony and maximum likelihood methods, phylogenetic analyses based on the mtDNA cytochrome *b* gene

have revealed significant molecular variation among *S. monticolus* and eight related species (*S. bairdi*, *S. bendirii*, *S. neomexicanus*, *S. ornatus*, *S. pacificus*, *S. palustris*, *S. sonomae* and *S. vagrans*).<sup>23</sup> Poorly resolved internal nodes within topologies suggest rapid diversification within this group. *S. monticolus* was not monophyletic under current taxonomic nomenclature, but formed two distinct clades: a continental clade and a coastal clade. The high degree of sequence similarity of the hantaviruses detected in *S. monticolus* from New Mexico and Colorado are consistent with their grouping into the (southern) continental clade.

In analyzing the cytochrome *b* and nicotinamide adenine dinucleotide dehydrogenase 4 mtDNA genes, eight members of the *S. cinereus* group (*S. camtschatica*, *S. cinereus*, *S. haydeni*, *S. jacksoni*, *S. portenkoi*, *S. preblei*, *S. pribilofensis*, and *S. ugunyak*) and *S. longirostris* were placed into northern and southern clades, with *S. cinereus* and *S. longirostris* in the latter.<sup>24</sup> A recent analysis estimates the divergence time at approximately 10 million years before present.<sup>25</sup>

The newly identified hantaviruses in the masked shrew and

dusky shrew in the United States, when considered within the context of the recently identified hantavirus in the northern short-tailed shrew,<sup>11</sup> heralds the discovery of additional hantaviruses in shrews within North America and beyond. Studies, now underway, will examine the epizootiology and molecular phylogeny of JMSV and ARR V throughout the respective geographic ranges of *S. monticolus* and *S. cinereus*. Investigations will also focus on the possibility of genetic reassortment among hantaviruses harbored by sympatric *Sorex* species. Finally, the isolation of these novel hantaviruses in cell culture will facilitate investigations aimed at determining their definitive host associations, as well as their pathogenicity for humans.

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